XXVII. STUDIES IN THE METABOLISM OF TISSUES GROWING *IN VITRO*.

III. CYANIC ACID AS A POSSIBLE PRECURSOR OF THE AMMONIA AND UREA FORMED BY EMBRYO KIDNEY TISSUE.

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It has already been shown [Holmes and Watchorn, 1927; Watchorn and Holmes, 1927] that embryonic kidney tissue of the rat growing *in vitro* is capable of forming both ammonia and urea, while under the same conditions non-growing tissue is inactive in this respect. For the sake of clearness, it is necessary to recapitulate this work to some extent, but for details earlier papers should be consulted. Ammonia and urea are estimated by a modification of Stanford's [1923] method, as previously described. The medium employed is an embryonic tissue extract, made up in Pannett and Compton's [1924] modification of Ringer's solution. The controls used are the following:

(A) 2 cc. of medium, kept at 0° for 48 hours;

(B) 2 cc. of medium, plus the same amount of embryo kidney tissue as is used in the experimental flasks; this is also kept at 0° for 48 hours;

(C) 2 cc. of medium incubated for 48 hours.

The experimental preparations are also incubated for 48 hours and consist of 2 cc. of medium plus embryo kidney tissue. From control B the amounts of ammonia and urea in the medium and kidney tissue at the beginning of the experiment can be determined, while C − A (the difference is usually small) allows for any ammonia and urea which may be formed by the medium itself during the 48 hours' incubation through autolytic or other processes. The total control is thus represented by B + (C − A). If the content of ammonia and urea in the experimental preparations is in excess of that in the total control, it is plain that the extra amounts have been formed by the activity of the tissue explants.

The growing tissue is supported upon cotton wool strands, while growth is prevented in the "resting" or non-growing preparations by lack of mechanical support. The depth of fluid even in this case is very small, but is just sufficient to float the minute fragments of tissue.

We have always found that the content of urea- and ammonia-nitrogen in the non-growing specimens is the same as that of the total control, that is to say these substances are not formed, under our conditions, by non-growing tissue. On the other hand the growing tissues form very considerable amounts

of both these substances. Quite early in the course of the work it became obvious that sometimes it was chiefly ammonia that was formed, and sometimes chiefly urea, although there was no reason to suppose that the conditions could have varied from one experiment to another. Moreover we have sometimes found that when one experimental series included two growing preparations, the total rise in ammonia- and urea-nitrogen might be the same in both of them, but in one case it might be predominantly urea and in the other predominantly ammonia formation which accounted for the rise. The medium on the other hand would be identical in the two cases, and the kidneys taken from embryos of the same litter, so that conditions would not differ to any appreciable extent. The following example is taken from an experiment giving this type of result:

	Total control mg.	1st grower mg.	2nd grower mg.
NH_3-N	0·060	0·073	0·084
Urea-N	0·027	0·054	0·040
NH_3 + Urea-N	0·087	0·127	0·124

It is quite certain that rat kidney tissue does not contain urease, so that in no case can the ammonia be formed from urea, and, on the other hand, we have never obtained any results suggesting that the tissue is capable of converting ammonia into urea. In order to test this point we have carried out experiments in which ammonia has been added to the medium. In no case, either in floating or growing preparations, has there been any utilisation of the ammonia to form urea.

It seems therefore, that we cannot account for the apparently alternative appearance of urea and ammonia during growth by assuming that either of these substances, once formed, can be converted into the other.

The simplest explanation of the results is that there is a common precursor of urea and ammonia, which may be easily converted into either of these, and which is made by the growing tissues during the breakdown of nitrogenous substances.

Werner [1923] and Fearon and Montgomery [1924] have suggested that cyanic acid may arise in the animal body as a result of the deamination of amino-acids, and that this could then give rise (as it is, of course, well known to do *in vitro*) to both ammonia and urea. Fearon and Montgomery have shown that cyanate may be formed during the oxidation of amino-acids *in vitro* (particularly in the presence of carbon compounds), but the evidence for its formation in the animal body is very slender.

However, if Werner's theories are correct, we may imagine that the growing preparations of kidney tissue can form cyanic acid during the deamination of the protein derivatives contained in the medium. The non-growing tissues do not carry out these deaminations to any extent, but if supplied with cyanic acid from outside they should produce ammonia and urea from it, so that a non-growing preparation to which cyanic acid had been

added might give the same type of result as a growing preparation with an ordinary medium. A series of experiments was undertaken to test this point.

Neutralised potassium cyanate (p_H 7·2), which contains, of course, a considerable proportion of free cyanic acid, was used. The Ringer's solution containing cyanate was sterilised by filtration through a candle, in order to avoid breakdown of the cyanate. The final concentration was such that about 0·05 or 0·06 mg. of cyanate-nitrogen was present in 2 cc. medium. The best results are obtained with embryos two or three days before their expected birth. It was very necessary to show that the cyanate in this concentration was not toxic to the tissues, as dead tissue would probably give rise to ammonia and urea by autolysis. Many microscopical examinations were therefore carried out, and it is safe to say that the cyanate did not inhibit the growth of embryonic kidney tissue and it is very improbable that it was appreciably toxic at this dilution. The objection of the extreme toxicity of cyanate has often been brought forward against the Werner and Fearon theory. This objection is plainly not valid, and it must be pointed out that the concentration of cyanic acid in our medium (7·5–9·0 mg. cyanic acid per 100 cc.) is considerably greater than that supposed by Montgomery [1925] to be present in blood.

Table I.

	Ammonia-N		Urea-N		Total urea-N + NH_3-N	
Exp. No.	Control mg.	Resting tissue mg.	Control mg.	Resting tissue mg.	Control mg.	Resting tissue mg.
54	0·034	0·045	0·016	0·016	0·050	0·061
59	0·026	0·021	0·021	0·052	0·050	0·073
60	0·036	0·054*	0·040	—	0·076	—
		0·050		0·048		0·098
66	0·030	0·048	0·016	0·021	0·046	0·069
		0·043		0·019		0·062
69	0·026	0·039	0·028	0·028	0·054	0·067
		0·033		0·050		0·083
70	0·034	0·035	0·032	0·050	0·066	0·085
		0·048		0·065		0·113
72	0·028	0·054	0·042	0·044	0·070	0·098
		0·060		—		—

* Where two sets of figures are given, these refer to two separate preparations in the same experimental series, the control therefore being the same for both.

The results are given in Table I, and it can be seen that in the presence of non-growing embryonic kidney tissue the added cyanate is partly broken down, yielding ammonia or urea, or a mixture of the two, thus giving results exactly like those found in the case of growing tissue without added cyanate. When no tissue was present, and the medium incubated by itself (control C), there was not usually any perceptible breakdown of the cyanate, though this did occur on one or two occasions (these experiments are not quoted in the table). It is therefore undoubtedly true that the kidney tissue can catalyse the breakdown of the cyanate in some way, though whether by direct enzymic action or by indirect physical means, such as local alterations of p_H in the medium, it is not possible to say.

No breakdown of cyanate occurs during the course of the estimations, provided that the temperature is not raised above 55° during the distillations.

Although these experiments cannot be held to prove that cyanic acid is formed by the nitrogenous metabolism of growing tissues, it can be said that if cyanic acid is formed during growth then the results which we have formerly described can be very satisfactorily explained.

Werner [1923] suggests that the following equation represents the formation of urea from cyanate:

$$2HN:CO + H_2O = CON_2H_4 + CO_2$$

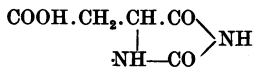
whereas in the presence of extra ammonia he thinks that more urea would be formed from the same amount of cyanate, the ammonia being utilised:

$$2HN:CO + 2NH_3 = 2CON_2H_4.$$

According to this theory, the tissues growing in embryo extract, if they were in fact forming cyanate, should utilise some of the ammonia already present to form urea. This, however, never occurred, even when extra ammonia was added to the medium. This does not show that cyanate is not formed during growth, as we were at first inclined to think, since when cyanate was actually added to the medium there was no sign of ammonia utilisation, nor was there anything to suggest that the proportion of urea formed depended upon the amount of ammonia present. Possibly the concentrations of ammonia used (about 3 mg. per 100 cc.) were not sufficient to affect the reaction. During life there may be local concentrations far greater than this, particularly in the functioning kidney and liver, but larger amounts, if added to the medium of cultures, would probably prevent growth.

The breakdown of 1: *hydantoinacetic acid.*

Some time ago Dakin [1908] described the appearance in the urine of the uramido-acid from phenylalanine when the latter was injected intravenously into a rabbit, and also of both the uramido- and the hydantoin derivatives when inactive tyrosine was fed to cats [Dakin, 1910, 1]. He suggested that urea might be formed directly from these without the intermediate formation of ammonia. He later [1926] pointed out that if cyanic acid were present in the body, it would probably react with amino-acids to give uramido-acids and hydantoin. When the uramido- and hydantoin derivatives of *dl*-phenylalanine and *dl*-leucine were fed to rabbits, they were excreted unchanged. However, when the derivatives of *d*-glutamic acid were fed, only a small proportion could be recovered, and the hydantoinacetic acid from *l*-aspartic acid was also mainly destroyed in the body.



Hydantoinacetic acid

It seems probable from Dakin's work that these substances are normally formed during metabolism and as their fate is of biochemical interest in connection with the cyanate theory of urea formation in the body, we prepared *l*-hydantoinacetic acid from *l*-aspartic acid and potassium cyanate according to the directions given by Dakin [1910, 2]. The hydantoin was then added to the medium in sufficient amounts to bring the final concentration to 0·06–0·08 %, and the Ringer's solution containing the hydantoin was sterilised by steaming three times. A few experiments only have been carried out, but they show quite definitely that the embryo kidney tissue can break down the hydantoin and form urea from it without necessarily showing any formation of extra ammonia. This breakdown may also occur in the medium when this is incubated by itself; the enzymes concerned are therefore probably not peculiar to the kidney, but can be extracted from other embryonic tissues. For instance, in one experimental series the urea-N of the total control should have been, judging by the controls kept at 0°, about 0·02 mg. whereas the urea-N in the incubated medium had increased to 0·03 mg. in one preparation and 0·054 mg. in another. A non-growing tissue preparation in the same series contained 0·043 mg. urea-nitrogen. No extra ammonia was found. In one experiment ammonia was produced, and not urea, the amounts being as follows: ammonia-N in total control 0·044 mg., and in two non-growing preparations 0·057 and 0·061 mg. respectively. It is therefore possible that cyanic acid and not urea itself can be first split off from the hydantoin, and give rise to either urea or ammonia.

It is not possible to say from these results whether hydantoin formation plays an important part in the production of urea from cyanate by kidney tissue.

Utilisation of urea by embryonic kidney tissue.

During the course of experiments with added cyanate we found that the growing preparations often showed a very considerable fall in urea content (Table II). It is not at all probable that this reaction would complicate the formation of urea from cyanic acid, supposing the latter to be formed during growth. It is reasonable to suppose that cyanic acid, if formed, would be broken down quickly, and would never accumulate to any extent. In this case the urea formation from cyanic acid would be the predominant reaction, and not the disappearance of urea that is noticeable when considerable amounts of cyanate have been added.

Table II.

Exp. No.	Total control Urea-N mg.	Non-growing tissue Urea-N mg.	Growing tissue Urea-N mg.
54	0·024	—	0·014
57	0·028	—	0·016
60	0·040	0·031	—
62	0·026	0·011	—
65	0·050	0·022	0·008
68	0·073	—	0·050
72	0·038	—	0·026

N.B. In all the above experiments cyanate was added to the medium.

The fall in urea content may be quite large, and when the fact that urea production is probably proceeding at the same time is taken into consideration, it will be seen that the amounts of urea utilised are very considerable. We know that the urea is not broken down to ammonia, so that it is probably taking part in a synthesis; as yet, however, we have no further knowledge of its fate.

Some of the examples given in Table II show a fall of urea-nitrogen in the non-growing tissue as well as in the growing. In the former it is more usual, as already pointed out, to find a rise in urea due to the breakdown of the cyanate, and the loss of urea is always greater when growth occurs. The incubated medium (control C) often showed a very slight decrease in its urea content when compared with the ice-chest medium (control A). That this decrease is real, and not an experimental error, is shown by the fact that on one occasion the drop in urea-N was considerable (over 0·01 mg.). Evidently then, the disappearance of urea does not entirely depend upon growth, or even upon the presence of embryo kidney tissue, but can be brought about to some extent by the embryo extract used for the medium.

The disappearance of urea during growth had previously been noticed [Watchorn and Holmes, 1927] when the medium contained additional glucose, though never when the plain embryo extract was used.

Summary.

(1) The possibility that cyanic acid may be the precursor of ammonia and urea formed by growing kidney tissue has been discussed, and experiments have been described to test this point.

(2) Cyanic acid in the presence of embryo kidney tissue is broken down to ammonia and urea, and the tissue catalyses the reaction.

(3) Cyanic acid does not appear to have a toxic effect upon the tissue, when it is present in amounts up to 9·0 mg. per 100 cc. of medium.

(4) *l*-Hydantoinacetic acid, which might arise in the body as the result of a reaction between *l*-aspartic acid and cyanic acid, is also broken down by embryonic tissue, and gives rise to urea and ammonia.

(5) In the presence of cyanate, urea may disappear from the cultures. This is particularly the case when the tissue is actively growing.

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